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# Mechanism of ATP Inhibition of Mammalian Type I DNA Topoisomerase: DNA Binding, Cleavage, and Rejoining Are Insensitive to ATP†

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ABSTRACT: A general, unrefined mechanism of type I DNA topoisomerase action involves several steps including DNA binding, single-strand scission, strand passage, resealing, and, possibly, readoption of an active enzyme conformation. None of these steps requires an energy cofactor; however, we have shown previously that several mammalian type I topoisomerases are, in fact, inhibited by ATP. In this study, we wanted to examine which steps in the gross topoisomerase mechanism were sensitive or insensitive to ATP. Nitrocellulose filter binding experiments showed that ATP did not interfere with the binding of DNA by the enzyme and that ATP binding by topoisomerase was 5-fold greater in the presence of DNA than in its absence. Agarose gel electrophoresis in the presence or absence of ethidium bromide indicated that resealing was unaffected by added ATP. The addition of the adenine nucleotide did not alter the pattern of camptothecin-stimulated cleavage of DNA, indicating that strand scission was not the point of inhibition. To test whether strand passage or the readoption of an active conformation was an inhibited step, we used a unique DNA topoisomer as substrate. The results argued against readoption of an active enzyme conformation as an ATP-sensitive process.

DNA topoisomerases are enzymes that alter the topological state of DNA and thereby interconvert topological isomers via mechanisms that involve single-strand (type I enzyme) or double-strand (type II enzyme) breakage and rejoining. A large body of direct and indirect evidence has implicated the topoisomerases in almost every process involving DNA metabolism, including replication, transcription, repair, recombination, chromosome condensation, and viral packaging [reviewed by Wang (1985), Liu (1983), and Gellert (1981)]. Relatively little is known, however, about the regulation of topoisomerase activity. The expression of DNA gyrase seems to be regulated by the overall superhelicity of the bacterial chromosome; when the degree of supertwisting of the genome is low, DNA gyrase production is increased (Menzel & Gellert, 1983). This increase in gyrase is apparently necessary to counterbalance the relaxing efforts of the bacterial type I topoisomerase ($\omega$ protein). This topoisomerase interplay is further supported by genetic evidence. A number of bacterial mutants that have deleted the *topA* gene are able to survive due to compensatory mutations in *gyrA* or *gyrB* that reduce gyrase activity (DiNardo et al., 1982; Pruss et al., 1982).

Even less is known about the regulation of the eukaryotic topoisomerases. In vitro experiments have shown that histone H1 and several high mobility group (HMG)[1] proteins can stimulate topoisomerase I (Javaherian & Liu, 1983), while epidermal growth factor can stimulate topoisomerase II activity in human fibroblasts and Swiss/3T3 mouse fibroblasts (Miskimins et al., 1983). In addition, several modifications of topoisomerases lead to alterations of enzyme activity. For example, ADP-ribosylation inactivates (Ferro et al., 1983) and phosphorylation stimulates (Mills et al., 1982) topoisomerase I while phosphorylation of *Drosophila* topoisomerase II increases enzyme activity (Sander et al., 1984).

Although the type I topoisomerases do not require any energy source, we have recently reported an ATP effect which

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[1] Abbreviations: HMG, high mobility group; topo, topoisomerase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; bp, base pair(s); SDS, sodium dodecyl sulfate.

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may be involved in regulating mammalian topoisomerase action (Castora et al., 1985; Castora & Kelly, 1986). The inhibition of topoisomerase I by physiological concentrations of ATP was observed with enzyme isolated from human leukemia, calf thymus, and HeLa cells (Castora & Kelly, 1986). A related observation was made by others using HeLa cell enzyme (Low & Holden, 1985). In this study, we have investigated the mechanism of inhibition of topoisomerase I by ATP. The broad mechanism by which mammalian type I topoisomerases are proposed to operate has been suggested by the work from a number of laboratories (Champoux & Dulbecco, 1972; Champoux, 1977; Keller et al., 1977; Been & Champoux, 1981; Halligan et al., 1982). It involves the following unrefined steps: (a) binding of the enzyme to DNA; (b) single-strand scission and restraint of free rotation; (c) passing of an intact strand through the break; (d) resealing; and, possibly, (e) the enzyme readopting an active conformation. Whereas it has been established that hydrolysis of ATP is required for type II topoisomerases to reassume an active conformation after a single round of breaking and rejoining (Sugino et al., 1978), similar conformational changes have not been documented for type I topoisomerases, although kinetic data of Pulleyblank and Ellison (1982) do suggest that the enzyme occurs in different conformations during the nicking and resealing steps. Presumably the topoisomerase I undergoes at least one conformational change when it must accommodate passing the intact strand through the break without allowing free rotation after which it ligates the broken ends. This implies that there is a cyclical return to the starting or active conformation when the cleaving/passing/resealing process is repeated again and again.

In this study, we wanted to dissect the overall mechanism of topoisomerase action into broad steps with the view of eliminating one or more of these general steps as the ATP-sensitive one, and possibly identifying an overall ATP-sensitive process. Such a strategy would also reduce the effort necessary to elucidate the details of the nucleotide inhibition when a more refined mechanism of topoisomerase action is examined. Our progress thus far suggests that DNA binding, strand cleavage, and resealing may be uninfluenced by the presence of ATP.

## Materials and Methods

Hydroxyapatite (Bio-Gel HTP) and Bio-Rex 70 were purchased from Bio-Rad. Heparin–Sepharose 6B was from Pharmacia. [$\gamma$-$^{32}$P]ATP (specific activity 200 Ci/mmol) and [$\alpha$-$^{32}$P]dATP (specific activity 400 Ci/mmol) were from New England Nuclear. Adenosine 5′-triphosphate was from Sigma. Camptothecin (NSC94600) was from the National Cancer Institute. The drug was dissolved in dimethyl sulfoxide to 10 mM and stored at −20 °C. Supercoiled plasmid pBR322 was grown in *Escherichia coli* strain N100 and purified by two cesium chloride/ethidium bromide centrifugations. DNA topoisomerase I from calf thymus was purified to homogeneity according to Schmitt et al. (1984). Active fractions from the last column (Bio-Rex 70) were dialyzed into storage buffer containing 40 mM Tris-HCl, pH 7.5, 10 mM $\beta$-mercaptoethanol, and 50% glycerol and kept at −20 °C.

*Topoisomerase Assay.* Standard topoisomerase relaxation assays and gel electrophoresis were performed as described by Lazarus et al. (1987). One unit of relaxing activity removes 50% of the supercoils from the substrate in 30 min at 30 °C.

*Reversibility Assay.* Spun columns were prepared with Sephadex G-50 in 1-mL disposable syringes (Maniatis et al., 1982) and were equilibrated with assay buffer containing 40 mM Tris-HCl, pH 7.0, 60 mM KCl, 10 mM $MgCl_2$, and 1 mM EDTA. Topoisomerase assays with and without 5 mM ATP were incubated at 30 °C for 30 min, applied to separate columns, and spun 4 min at 3000 rpm in a benchtop centrifuge. Aliquots of the effluent were then incubated at 30 °C for 30 min and the products separated by agarose gel electrophoresis.

*Binding Assay.* Linear, end-labeled [$^{32}$P]DNA (specific activity $2 \times 10^6$ cpm/$\mu$g) was prepared from *Hind*III- or *Eco*RI-digested plasmid pBR322. The endonuclease-generated ends were filled in by using the Klenow polymerase, dNTPs, and [$\alpha$-$^{32}$P]dATP as described (Maniatis et al., 1982). Different amounts of topoisomerase I (from 0 to 3.2 ng) were mixed with $^{32}$P-labeled DNA (50 ng) or [$\gamma$-$^{32}$P]ATP (specific activity 200 Ci/mmol), adjusted to 5 mM final concentration with unlabeled ATP in assay buffer (final volume 60 $\mu$L). These assays were incubated at 30 °C for 6 min and stopped by dilution with 120 $\mu$L of assay buffer. The solutions were applied to nitrocellulose filter papers (Schleicher & Schuell) that had been presoaked in assay buffer for 20 min and then rinsed once with assay buffer (Jones & Berg, 1966). The filters containing adsorbed protein and/or DNA/protein complexes were rinsed once with 10 mL of assay buffer, dried, and counted. The data were plotted after subtraction of background from control samples prepared in the absence of protein.

*Strand Breaking Assay.* Uniquely end-labeled DNA was prepared as follows: *Hind*III-linearized pBR322 was labeled at its 3′ ends with the large fragment of *E. coli* DNA polymerase I and [$\alpha$-$^{32}$P]dATP as above and then digested with *Eco*RI to release a small 31 bp fragment. DNA was precipitated with ethanol and suspended in 10 mM Tris/1 mM EDTA, pH 7.0. The labeled DNA was passed through a spun column to remove low molecular weight species and then ethanol precipitated. A topoisomerase cleavage assay stimulated by camptothecin was performed according to Liu et al. (1983). The assay contained 50 ng of labeled DNA, 16 ng of topoisomerase I, and up to 100 $\mu$M camptothecin. When present, the ATP concentration was 10 mM. After incubation at 30 °C for 30 min, the assays were mixed with alkaline stop solution and electrophoresed on neutral agarose gels (Hsiang et al., 1985). The gels were dried and autoradiographed.

*Strand Sealing Assay.* Topoisomerase assays containing different concentrations of ATP were incubated at 30 °C for 30 min and loaded on 0.7% agarose gels which either lacked or contained 1 $\mu$g/mL ethidium bromide. The gels were electrophoresed 16 h at 40 V, and DNA bands were visualized and photographed under UV illumination. Non-ethidium gels were stained for 30 min with ethidium bromide before being photographed.

*Strand Passing Assay.* pBR322 DNA of unique topological winding number was prepared by topoisomerase I relaxation of supercoiled pBR322. DNA containing seven superhelical turns was isolated from an agarose gel by electroelution. This unique topoisomer was used as substrate in a standard assay mixture plus or minus ATP. The products were separated by agarose gel electrophoresis, transferred to nitrocellulose, and visualized by Southern hybridization using nick-translated pBR322 as probe.

## Results

*Inhibition by ATP Is a Reversible Process.* In order to elucidate the effect of ATP and its analogues upon topoisomerase I activity, we first determined whether the nucleotide was interacting with the enzyme in a reversible or irreversible manner. In order to establish the nature of this interaction, we tried three approaches.

In the first two experiments, removal of ATP from the samples was accomplished by dialysis or centrifugation in

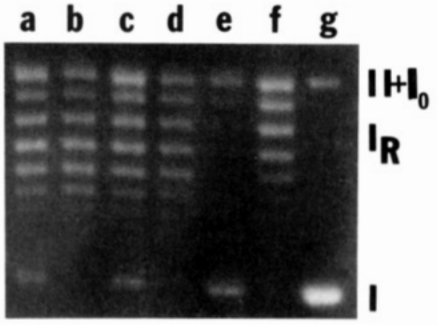


FIGURE 1: Reversibility of the ATP inhibition of topoisomerase I. Lane a, aliquot of ATP-inhibited reaction passed twice through the spun column; lane b, aliquot of an uninhibited reaction passed twice through the spun column; lane c, aliquot of ATP-inhibited reaction passed once through the spun column; lane d, aliquot of an uninhibited reaction passed once through the spun column; lane e, standard topoisomerase assay (2 units of enzyme) plus 5 mM ATP; lane f, standard topoisomerase assay; lane g, control pBR322 (no enzyme added). The DNA bands identified as I, $I_0$, $I_R$, and II are supercoiled, fully relaxed, partly relaxed, and nicked circular molecules, respectively.

Amicon concentrating devices. These two approaches resulted in loss of topoisomerase activity at a rate equal to the removal of ATP. In a third approach, ATP removal was effected with a 1-mL spun column containing Sephadex G-50 (Maniatis et al., 1982). Figure 1 shows results of topoisomerase I assays in the presence and absence of ATP either before or after being passed thorugh the column. Lane f shows that the topoisomerase relaxed the highly supercoiled substrate, generating a ladder of DNA species possessing only a small number of supertwists. When ATP was present, there was a substantial reduction in relaxation activity (lane e). After being normalized by densitometry for variations in DNA loaded, the assay in lane e represented a greater than 80% inhibition of topoisomerase activity. When these same assays were passed through a spun column and reincubated at 30 °C for 30 min, it was found that the uninhibited sample retained all of its activity (lane d) and the ATP-inhibited sample regained 94% of its activity (lane c). A second passage through the column gave similar results, with the ATP-treated sample exhibiting 96% of its original activity. From these results, it was clear that removal of the nucleotide resulted in restoration of relaxation activity, thereby characterizing the inhibition of topoisomerase I by ATP as reversible. We next decided to examine where in the mechanism of action of topoisomerase I the ATP could be exerting its inhibitory effect.

*ATP Does Not Reduce the Binding of DNA by Topoisomerase.* To test whether the ATP was affecting the binding of DNA by the enzyme, nitrocellulose filter binding assays using linearized, radiolabeled pBR322 as substrate were performed. In the absence of ATP, increasing levels of topoisomerase increasingly bound substrate until a plateau was reached at about 1.2 ng/assay (Figure 2A). This corresponded to a level of 12 fmol of topoisomerase/2.4 fmol of DNA, or 5 mol of enzyme/mol of DNA, which agrees well with earlier results (Prell & Vosberg, 1980). When ATP was included at concentrations as high as 10 mM, there was no alteration in the DNA binding profile of the topoisomerase (Figure 2A). Independent topoisomerase gel assays showed that this level of ATP was sufficient to completely inhibit relaxation catalyzed by up to 1.6 ng of enzyme. Thus, the presence of ATP did not impair the binding of the topoisomerase with its substrate.

*Binding of ATP Occurs Preferentially in the Presence of DNA.* If the high concentrations of ATP did not interfere with binding of the substrate by the enzyme, was ATP binding to

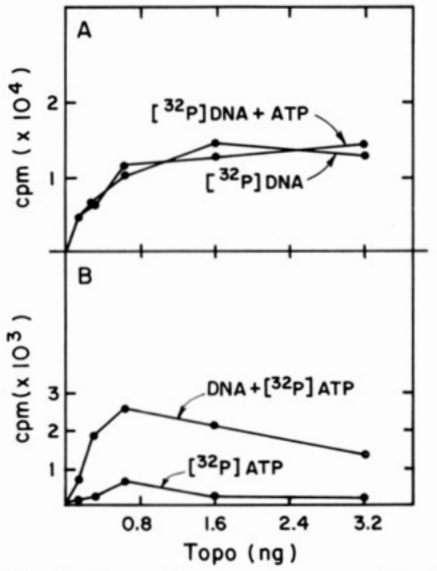


FIGURE 2: Filter binding profiles of topoisomerase with substrate and ATP. (A) The binding curve of increasing concentrations of topoisomerase with radiolabeled, linear pBR322 in the presence and absence of ATP. (B) The binding curve of topoisomerase with $^{32}P$-labeled ATP (specific activity 200 Ci/mmol) in the presence and absence of unlabeled, linear pBR322.

the topoisomerase at all? We addressed this question by repeating the nitrocellulose filter binding experiment using $^{32}P$-labeled ATP (200 Ci/mmol) and unlabeled, linear pBR322. The resulting profile (Figure 2B) showed that the topoisomerase weakly bound ATP (0.18 mol of ATP/mol of topoisomerase). When substrate DNA was present, the affinity of the enzyme for ATP was increased 5-fold to 0.9 mol of ATP/mol of topoisomerase (Figure 2B). This result was consistent with a preliminary microdialysis experiment which showed an increased retention of ATP by the topoisomerase only in the presence of DNA. This increased binding of ATP in the presence of DNA suggested that the enzyme may be undergoing a conformational change which enhances the interaction of ATP with the topoisomerase. Furthermore, the data indicated that each topoisomerase molecule may have a single nucleotide binding site and that the binding of one ATP molecule per molecule of enzyme would be sufficient to inactivate the topoisomerase relaxation activity. The reason for the decrease in bound counts with increased amounts of topoisomerase in panel B is not clear to us, but similar behavior has been reported by others (Prell & Vosberg, 1980).

These binding results suggested that the presence of ATP in sufficient concentrations to inhibit relaxation did not inhibit the association of the topoisomerase with its substrate. In addition, they indicated that ATP bound directly to the enzyme and that this binding was stimulated as much as 5-fold when substrate was present.

*ATP Does Not Interfere with Resealing of Topoisomerase-Induced Breaks.* The inhibition of topoisomerase activity brought about by addition of ATP was normally assessed by separating the products of the assay by agarose gel electrophoresis. A typical gel is shown in Figure 3A. Here, the removal of supercoils from substrate DNA was clearly seen in lane b. Full or limit relaxation by topoisomerase always resulted in a ladder of bands containing only a few supercoils. This distribution is dependent on the temperature and ionic conditions at which the assay and electrophoresis were performed. Although there is frequently a significant enrichment of the form $I_0$ DNA, there will always be species with some small number of supercoils present after enzymatic relaxation.

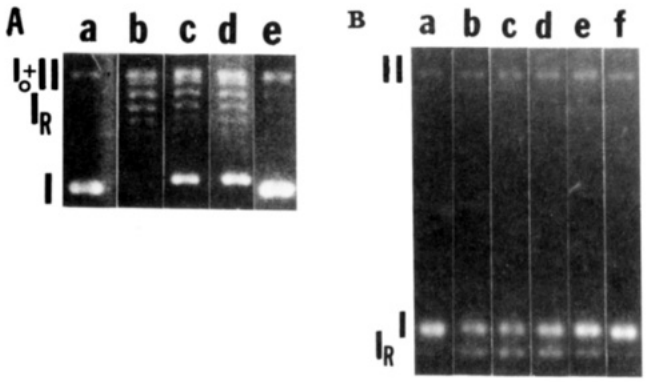


FIGURE 3: Agarose gel electrophoresis of topoisomerase products obtained in the presence or absence of added ATP. (A) A standard non-ethidium bromide gel of assays performed in the presence of increasing amounts of ATP: lane a, pBR322 control (no enzyme added); lane b, plus 2 units of topoisomerase I; lane c, same as (b) plus 0.5 mM ATP; lane d, same as (b) plus 1.0 mM ATP; lane e, same as (b) plus 5.0 mM ATP. (B) An agarose gel run in the presence of 1 $\mu$g/mL ethidium bromide: lane a, pBR322 control (no enzyme added); lane b, plus 1 unit of topoisomerase; lane c, same as (b) plus 0.25 mM ATP; lane d, same as (b) plus 0.5 mM ATP; lane e, same as (b) plus 1.0 mM ATP; lane f, same as (b) plus 2.5 mM ATP. The DNA band assignments are the same as in Figure 1.

This relaxation was reduced as the ATP concentration increased from 0.5 to 5.0 mM (lanes c–e). Although there was a significant reduction in relaxation as ATP levels were raised, this inhibition did not lead to an increase in open circular or nicked DNA. If the ATP interfered with the ability of the enzyme to reseal the broken DNA strand, then an accumulation of nicked DNA would have been expected. This was not evident in Figure 3A. However, nicked DNA and completely relaxed DNA comigrate in this gel system. In order to more accurately assess the resealing of breaks, gels containing ethidium bromide were used to allow complete separation of form $I_0$ and form II DNA.

Figure 3B shows the results of such assays performed in the presence and absence of inhibitory levels of ATP. Lane b contained topoisomerase in the absence of ATP. There was approximately 1 unit of relaxation activity which gave rise to the rapidly moving band ($I_R$) ahead of supercoiled DNA. Adding ATP decreased activity, this decrease being most evident at 2.5 mM (lane e) and 5 mM (lane f). At 5 mM ATP, relaxation was completely inhibited, and the DNA (lane f) resembled the control (lane a). It is important to note that the inhibited samples (lanes c–f) showed no increase in nicked DNA (form II). Quantitation by densitometry indicated that no increase in the relative amount of form II DNA occurred.

*Added ATP Does Not Affect the Products or Rate of Cleavage of DNA by Topoisomerase I.* Although the instantaneous fraction of topoisomerase covalently linked to the broken DNA strand is small, this fraction can be increased substantially if camptothecin is added to the assay mix (Hsiang et al., 1985). This drug traps the topoisomerase in a cleavable complex which leads to DNA fragmentation upon protein denaturation with SDS. As seen in Figure 4A, enzyme treatment of labeled DNA in the absence of camptothecin resulted in little if any DNA cleavage (lane b) which was unaffected by including camptothecin (lane c) or 10 mM ATP (lane d) alone. However, when camptothecin was added to enzyme-treated samples, the cleavable complex was trapped, and the DNA was extensively degraded (lane e). In the presence of 10 mM ATP, there was no change in the camptothecin-induced cleavage of the DNA (lane f). The labeled substrate was extensively degraded whether ATP was present at concentrations that inhibited relaxation or not. Complete

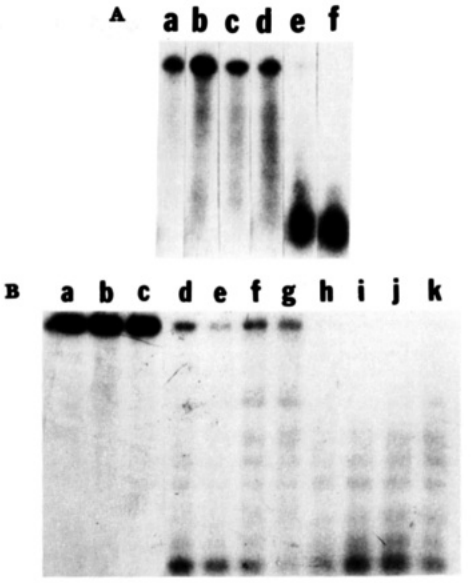


FIGURE 4: Strand cleavage by topoisomerase I in the presence of ATP and camptothecin. (A) End-point determinations: lane a, $^{32}$P-labeled pBR322; lane b, plus topoisomerase I (16 ng); lane c, plus 10 mM ATP; lane d, plus topoisomerase plus 10 mM ATP; lane e, same as (b) plus 100 $\mu$M camptothecin; lane f, same as (d) plus 100 $\mu$M camptothecin. (B) Intermediate topoisomerase cleavage products: lane a, control; lane b, plus ATP; lane c, plus ATP and 50 $\mu$M camptothecin; lanes d–g, plus topoisomerase and 50 $\mu$M camptothecin for 1, 2, 5, and 10 min, respectively; lanes h–k, same as (d–g) plus 10 mM ATP.

Table I: Effect of Camptothecin on Binding of ATP by Topoisomerase

| amount of topo (fmol) | binding[a] (cpm) | |
|---|---|---|
| | −drug | +drug |
| 2 | 552 | 605 |
| 4 | 606 | 750 |
| 10 | 911 | 1922 |
| 40 | 1336 | 1905 |

[a] $^{32}$P-Labeled ATP was adjusted to a final concentration of 5 mM with unlabeled ATP. The camptothecin concentration was 50 $\mu$M when present. Details are given under Materials and Methods.

degradation, which is a measure of the reaction end point, was unaffected by added ATP, and, as Figure 4B shows, intermediate levels of degradation were not reduced as well. In fact, there was even a slight increase in the amount of these intermediates in the presence of ATP. This was evident from the increased quantity of more rapidly migrating DNA fragments and the disappearance of linear starting material (cf. lanes d–g and h–k).

In addition, ATP did not affect the kinetics of this cleavage process. With 50 $\mu$M camptothecin in the assay, the final distribution of these intermediates was seen as early as 60 s. With ATP included, the pattern of the intermediate bands after 60 s was unchanged for up to 10 min of reaction time (cf. lanes h and k). Thus, the speed with which topoisomerase cleaved DNA strands was uninfluenced by ATP.

Camptothecin did not interfere with the interaction of ATP with the enzyme. This was shown by filter binding experiments performed as described in Figure 2. When 50 $\mu$M camptothecin was present, ATP was still bound by the topoisomerase to a degree roughly equivalent to the binding of ATP in the absence of camptothecin (Table I).

Thus, the absence of any inhibitory effect on camptothecin-stimulated cleavage was not due to the drug interfering or competing with the binding of ATP to the topoisomerase. This further argued that a step subsequent to DNA/enzyme

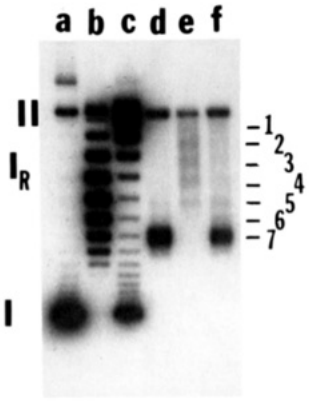


FIGURE 5: Unique topoisomer assays. This is an autoradiograph of topoisomerase products electrophoresed, transferred to nitrocellulose, and probed with labeled pBR322. Lane a, control pBR322 (no enzyme added); lane b, plus 4 fmol of topoisomerase; lane c, plus topoisomerase plus 5.0 mM ATP; lane d, unique topoisomer of pBR322 possessing seven supercoils; lane e, plus 4 fmol of topoisomerase; lane f, plus topoisomerase plus 5.0 mM ATP.

binding and strand breaking was sensitive to ATP inhibition.

*ATP May Influence Some Aspect of the Strand Passing Ability of Topoisomerase I.* Using a substrate which contained a defined number of superhelical turns allowed us to test the other possibilities: Was strand passage or was readoption of an active enzyme conformation a point of inhibition?

Mechanistically, if ATP inhibited the readoption of an active conformation after a single round of catalysis, then a population of DNA molecules with a well-defined number of supercoils would be expected to undergo a change in linking number of one unit; e.g., a substrate with seven supercoils would be changed to one with six supercoils. If multiple strand passing events occurred between the breaking and resealing events (Kirkegaard et al., 1984), the presumed conformational change would occur after a linking number change of more than 1. This would result in even a greater change in the final topoisomer distribution from its starting position. Further increases in linking number (removal of supercoils) would be inhibited by ATP blocking the ability of the enzyme to readopt an active conformation.

On the other hand, if strand passage or some aspect thereof were the site of ATP inhibition, then no change in the linking number of the substrate would occur. When the enzyme bound DNA, cleaved a single strand, and was unable to pass an intact strand through the enzyme-generated break, resealing would occur to yield DNA with the same number of supercoils.

Agarose gel electrophoresis was used to discriminate between these two possibilities. In order to detect small quantities of DNA, the products of topoisomerase assays were separated by gel electrophoresis, transferred to nitrocellulose, and visualized by hybridization to radiolabeled pBR322. To normalize for differences in DNA added to each lane and retained on the nitrocellulose filter, the autoradiographs were quantitated by densitometry. Excess enzyme (>4 units) was added in order to get sufficient relaxation of the slightly supercoiled substrate used in lanes d–f of Figure 5. This amount of enzyme led to removal of most of the supercoils (lane b) from the highly supertwisted starting substrate (lane a). When 5 mM ATP was included in the assays with the highly supercoiled substrate and excess topoisomerase, there was a reduction in relaxation activity of 75% (to 1 unit, lane c). Lane d shows the untreated, unique topological DNA species possessing seven negative supercoils. When treated with topoisomerase, this substrate was relaxed in steps of one to give the distribution shown in lane e. The relaxation of the unique topoisomer was complete, as all starting material was removed (lane e). By comparison, relaxation was almost completely inhibited by the addition of ATP (lane f). Approximately 95% of the substrate remained unaltered. The small amount of relaxation detected was most likely due to the excess enzyme used in this assay, some of which escaped ATP inhibition. This was consistent with our previous finding that even at 5 mM ATP, there was some small amount of relaxation observed with elevated levels (>2 units) of topoisomerase (Castora & Kelly, 1986).

## Discussion

Previously we have shown that the ATP-independent, type I topoisomerases from human leukemia, HeLa, and calf thymus cells are inhibited by physiological concentrations of ATP (Castora et al., 1985; Castora & Kelly, 1986). In this paper, we have undertaken to examine where in the topoisomerase I mechanism ATP may be exerting its effect. The proposed general mechanism involves DNA binding, strand cleavage, strand passage, resealing, and readoption of an active conformation. The detailed molecular events occurring during each of these general steps are for the most part unknown. Therefore, it is possible that the kinetic scheme described may not be complete in all respects and one or more of these presumed steps may not be completely correct. Enzyme mechanisms in general may be very complex, and the variety of reactions catalyzed and activities possessed by DNA topoisomerases only multiply the difficulty associated with unraveling the mechanistic details at the molecular level. However, as a point of embarking on the elucidation of the mechanism of ATP inhibition of topoisomerase action and as a possible means for illuminating some of these molecular details, we have attempted to identify the ATP-sensitive and the ATP-insensitive stages of the unrefined topoisomerase mechanism. Our results indicate the following.

(1) Binding of DNA by topoisomerase is unimpeded by added ATP, even though ATP does bind to the enzyme. In addition, binding of ATP is increased 5-fold in the presence of substrate DNA.

(2) The enzyme is able to reseal breaks effectively in the presence or absence of ATP.

(3) Camptothecin-stimulated DNA cleavage by topoisomerase I occurs at the same rate and may even be stimulated, not inhibited, in the presence of ATP. Furthermore, camptothecin does not interfere with the ability of ATP to bind to the topoisomerase.

(4) In the presence of ATP, a well-defined topological substrate yields products consistent with a mechanism of strand passing inhibition but inconsistent with one involving inhibition of readoption of an active conformation.

The above results would appear to eliminate DNA binding, strand cleavage, and strand resealing as points of ATP inhibition and suggest that some step or steps subsequent to DNA cleavage would be responsible for the observed effects of ATP. An obvious candidate for this ATP-sensitive step may be the strand passage event or some aspect of that which may include multiple, as yet undefined, processes. Unfortunately, the strand passage event per se has not been amenable to experimental testing, and so direct observation of the effects of ATP cannot be unequivocally made. Although the strand passing event is experimentally difficult to observe directly, we have begun a variety of kinetic and thermodynamic experiments utilizing filter binding and fluorescence measurements in order elucidate this process.

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# Normal Cellular Ha *ras* p21 Protein Causes Local Disruption of Bilayer Phospholipid

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ABSTRACT: We have investigated the interactions of the p21 protein of c-Ha *ras* with its phospholipid environment. Gel filtration of detergent-"solubilized" p21 revealed that this preparation consisted of a mixture of multimolecular aggregates of protein and phospholipid and also a population of individual p21 molecules. Addition of 8 M urea to p21 preparations increased the solubility of the molecule in detergent solutions upon the removal of this denaturant. The progressive addition of the detergent cholate appeared to increase the efficiency of p21 preparations to bind GTP. This affinity for GTP was not removed even at high detergent concentrations, when delipification of the p21 was presumably effected. Modification of the composition of the phospholipid species surrounding the protein did not appear to alter its affinity for GTP. Electron spin resonance studies with membrane spin-labels indicated a perturbation of the bilayer extending to between 44 and 100 phospholipids surrounding the molecule. However, no evidence was found for any population of intimately bound phospholipid, which is seen as an annulus of about 30 lipids in transmembrane proteins such as $Ca^{2+}$-ATPase. From these results we propose that the Ha *ras* p21 protein has the ability to associate directly with the membrane in a manner clearly discernible from that of a transmembrane protein.

Many membrane-bound proteins rely on interactions with their phospholipid environment to maintain correct functional activity (Montgomery et al., 1985; Hesketh et al., 1976; Duran & Cabib, 1980). The *ras* gene product p21 is known to reside in the cytosolic face of the plasma mebmrane and to have an intrinsic GTPase activity (Willingham et al., 1980; Lacal et al., 1986). Numerous recent studies have shown that p21 mediates the transduction of signals from receptor molecules within the membrane, to regulate enzymes controlling the production of second messenger molecules, such as phospho-

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